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Ischemia-like oxygen and glucose deprivation mediates down-regulation of cell surface γ -aminobutyric acidB receptors via the endoplasmic reticulum (ER) stress-induced transcription factor CCAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP)

Maier, Patrick J ; Zemoura, Khaled ; Acuña, Mario A ; Yévenes, Gonzalo E ; Zeilhofer, Hanns Ulrich ; Benke, Dietmar

Abstract: Cerebral ischemia frequently leads to long-term disability and death. Excitotoxicity is believed to be the main cause for ischemia-induced neuronal death. Although a role of glutamate receptors in this process has been firmly established, the contribution of metabotropic GABAB receptors, which control excitatory neurotransmission, is less clear. A prominent characteristic of ischemic insults is endoplasmic reticulum (ER) stress associated with the up-regulation of the transcription factor CCAAT/enhancer-binding protein-homologous protein (CHOP). After inducing ER stress in cultured cortical neurons by sustained Ca^{2+} release from intracellular stores or by a brief episode of oxygen and glucose deprivation (in vitro model of cerebral ischemia), we observed an increased expression of CHOP accompanied by a strong reduction of cell surface GABAB receptors. Our results indicate that down-regulation of cell surface GABAB receptors is caused by the interaction of the receptors with CHOP in the ER. Binding of CHOP prevented heterodimerization of the receptor subunits GABAB1 and GABAB2 and subsequent forward trafficking of the receptors to the cell surface. The reduced level of cell surface receptors diminished GABAB receptor signaling and, thus, neuronal inhibition. These findings indicate that ischemia-mediated up-regulation of CHOP down-regulates cell surface GABAB receptors by preventing their trafficking from the ER to the plasma membrane. This mechanism leads to diminished neuronal inhibition and may contribute to excitotoxicity in cerebral ischemia.

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Ischemia-Like Oxygen and Glucose Deprivation Mediates Down-regulation of Cell Surface γ -Aminobutyric Acid_B Receptors via the ER Stress-Induced Transcription Factor CHOP

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Running title: CHOP-induced down-regulation of cell surface GABA_B receptors

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Keywords: Cell surface, cerebral ischemia, CHOP, endoplasmic reticulum (ER), endoplasmic reticulum stress, GABA receptor, G protein coupled receptors, neurons, trafficking.

Background: ER-stress associated with cerebral ischemia induces the expression of the transcription factor CHOP.

Results: Interaction with CHOP downregulates cell surface GABA_B receptors and thus GABA_B receptor-mediated neuronal inhibition.

Conclusion: Interaction of CHOP with GABA_B receptors in the ER prevents forward trafficking of the receptors.

Significance: This mechanism is expected to contribute to excitotoxicity in cerebral ischemia.

ABSTRACT

Cerebral ischemia frequently leads to long-term disability and death. Excitotoxicity is believed to be the main cause for ischemia-induced neuronal death. Although a role of glutamate receptors in this process has been firmly established, the contribution of metabotropic GABA_B receptors, which control excitatory neurotransmission, is less clear. A prominent characteristic of ischemic insults is endoplasmic reticulum (ER) stress associated with the up-regulation of the transcription factor CHOP. After inducing ER stress in cultured cortical neurons by sustained Ca²⁺-release from intracellular stores or by a brief episode of

oxygen and glucose deprivation (OGD, *in vitro* model of cerebral ischemia), we observed an increased expression of CHOP accompanied by a strong reduction of cell surface GABA_B receptors. Our results indicate that down-regulation of cell surface GABA_B receptors is caused by the interaction of the receptors with CHOP in the ER. Binding of CHOP prevented heterodimerization of the receptor subunits GABA_{B1} and GABA_{B2} and subsequent forward trafficking of the receptors to the cell surface. The reduced level of cell surface receptors diminished GABA_B receptor signaling and thus neuronal inhibition. These findings indicate that ischemia-mediated up-regulation of CHOP downregulates cell surface GABA_B receptors by preventing their trafficking from the ER to the plasma membrane. This mechanism leads to diminished neuronal inhibition and may contribute to excitotoxicity in cerebral ischemia.

GABA_B receptors are G_{i/o} protein coupled receptors composed of the two obligatory and functionally distinct subunits GABA_{B1} and GABA_{B2}. GABA_{B1} harbors the binding site for orthosteric ligands while GABA_{B2} contains a binding site for allosteric modulators, recruits the

G protein and is required for trafficking of the receptors from the endoplasmic reticulum (ER) to the plasma membrane (reviewed in (1)). GABA_{B1} contains an ER retention signal in the C-terminal domain, which retains unassembled GABA_{B1} in the ER. Heterodimerization with GABA_{B2} masks the ER retention signal and permits ER export of the receptor heterodimers (2-4). GABA_B receptors are abundantly expressed throughout the mammalian central nervous system where they mediate slow and persistent inhibition. According to their prominent role in regulating neuronal excitability, GABA_B receptors have been implicated in a variety of neurological disorders including cerebral ischemia.

In cerebral ischemia, excessive glutamatergic neurotransmission eventually leads to neuronal death (5). Decreased GABAergic activity appears to contribute to neuronal overexcitation (6) and there are indications that GABA_B receptors are down-regulated under ischemic conditions (7-10). This suggests that impaired GABA_B receptor signaling contributes to excitotoxicity. In line with these findings, enhancing GABA_B receptor activity during ischemic insults by application of the GABA_B receptor agonist baclofen has been reported to be neuroprotective *in vitro* and *in vivo* (10-19).

Cerebral ischemia induces ER stress, which is characterized by the accumulation of proteins in the ER, leading to the activation of several pathways to restore normal ER function (20). If, however, ER function is severely impaired and cellular homeostasis cannot be restored, apoptosis of the neuron is induced. ER stress triggers the activation and expression of a number of proteins, including up-regulation of the transcription factor CHOP (C/EBP-homologous protein, also known as C/EBP ζ , growth arrest- and DNA damage-inducible gene 153 [GADD153] or DNA-damage inducible transcript 3 [DDIT3]). CHOP belongs to the C/EBP transcription factor family and has been shown to trigger apoptosis (21).

Besides its function as a transcription factor, there is evidence that CHOP interacts with GABA_B receptors to regulate their cell surface expression. We previously showed that CHOP interacts with its C-terminal leucine zipper with the leucine zipper present in the C-terminal domain of GABA_{B2} and with its N-terminal domain with an as yet unidentified site in GABA_{B1} (22). Upon

coexpression in HEK 293 cells, CHOP induced the intracellular accumulation of GABA_B receptors and a significant reduction of cell surface receptors (22). However, the mechanism behind this down-regulation of cell surface receptors as well as its physiological role remained unclear. Because ischemic conditions cause ER stress and upregulates CHOP we aimed in the present study at elucidating the mechanism of CHOP-induced down-regulation of cell surface GABA_B receptors in neurons and its putative role in cerebral ischemia. We found that ER stress-induced CHOP interacts with GABA_B receptors in the ER to disrupt GABA_B receptor heterodimerization. This prevents forward trafficking of the receptors to the plasma membrane and thus leads to the down-regulation of cell surface receptors and reduced GABA_B receptor signaling. This mechanism is operative in an *in vitro* model of cerebral ischemia.

EXPERIMENTAL PROCEDURES

Antibodies – The following primary antibodies were used: rabbit GABA_{B1a,b} directed against the C-terminus of GABA_{B1} and rabbit GABA_{B1b} directed against the N-terminus of GABA_{B1b} (affinity-purified, 1:200 for immunofluorescence, 1:40 for *in situ* PLA; custom made by GenScript) (23) as well as rabbit GABA_{B2} directed against the N-terminus of GABA_{B2} (24) (affinity-purified, 1:500 for immunofluorescence, 1:40 for *in situ* PLA; custom made by GenScript), guinea pig GABA_{B2} (1:50; Chemicon International, # AB5394), rabbit GABA_A receptor α 1 subunit (25) (affinity purified 1:100 for *in situ* PLA), mouse GABA_A receptor β 2/3 subunits (25) (1:500 for *in situ* PLA), mouse GADD153/CHOP (1:100, Santa Cruz Biotechnology, # sc-7351), mouse GADD153/CHOP (1:100 for immunofluorescence, 1:30 for *in situ* PLA; Cell Signaling Technology, # 2895), rabbit Anti-MAP Kinase (ERK-1, ERK-2, 1:500; Sigma Aldrich, # M5670), mouse Anti-MAP Kinase Activated (diphosphorylated ERK-1&2, 1:250; Sigma Aldrich, # M9692), rabbit NeuN (1:500; Millipore AG, # ABN78), goat PDI (1:150; Santa Cruz Biotechnology, # sc-17222), mouse GM130 (1:1000; Abcam, # ab32337), rabbit HA (1:200; Santa Cruz Biotechnology, # sc-805), rabbit c-myc (1:500; Santa Cruz Biotechnology, # sc-789). Secondary antibodies were either coupled to Alexa Fluor 488 (1:1000,

Invitrogen), Cy-3 (1:500, Jackson ImmunoResearch) or Cy-5 (1:300, Jackson ImmunoResearch).

Plasmids – Plasmids containing full-length cDNA of human CHOP and deletion mutants of CHOP (C-terminal deletion (Δ LZ) and N-terminal deletion (Δ N)) were described previously (22). Plasmids containing wild type and mutant rat GABA_{B1} and rat GABA_{B2} were kindly provided by Bernhard Bettler (University of Basel, Switzerland) and described in (3).

Drugs – The following drugs were used: thapsigargin (1 μ M; Sigma Aldrich), (R)-baclofen (100 μ M; Tocris Bioscience), CPG 56999A (10 μ M; gift from Novartis, Basel, Switzerland).

Cell culture – Primary cortical neurons were prepared from E18 embryos of time-pregnant Wistar rats as described previously (26). Briefly, minced E8 cortex was incubated for 15 min with papain solution (0.5 mg/ml PBS, 1 mg/ml BSA, 10 mM glucose, 10 μ g/ml DNaseI), washed with Dulbecco's modified Eagle's Medium containing 10% fetal calf serum and titrated using a Pasteur pipette. Neurons were plated at a density of 120,000 cells onto poly-L lysine coverslips (12 mm) in 24-well plates and kept in culture at 37°C and 5% CO₂ for 11-15 days.

Neurons in neuron-glia co-cultures were transfected with plasmids using magnetofection exactly as described in (27). 60,000 cells were plated on 18 mm coverslips and kept in culture for 11-15 days at 37°C and 5% CO₂. Magnetofection was performed for 30 min on a pre-warmed magnetic plate.

Immunocytochemistry and Confocal Laser Scanning Microscopy – Multiplex-labeling immunocytochemistry was performed as described previously (26). For the visualization of cell surface GABA_B receptors, living neurons were incubated with primary antibodies for 2 h at 4°C in ACSF (2 mM CaCl₂, 2 mM MgCl₂, 30 mM L-glucose, 5 mM KCl, 119 mM NaCl, 25 mM HEPES, pH 7.4) containing 10% NGS (Normal Goat Serum). For staining of intracellular localized proteins, neurons were subsequently fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized for 6 min with 0.2 % Triton X-100. Neurons were then incubated with primary antibodies for 1 h (in PBS/10% NGS) at room temperature, washed four times for 5 min with PBS and incubated with secondary antibodies for 1

h. After four washes with PBS neurons were mounted in fluorescence mounting medium and analyzed by confocal laser scanning microscopy (LSM510 Meta; Zeiss). Images were acquired using a Zeiss 100x plan apochromat oil differential interference contrast objective (1.4 NA) at 512x512 pixel resolution for fluorescence intensity measurements or 1024x1024 pixel resolution for colocalization studies. For each neuron, 5 optical sections spaced by 0.4 μ m were taken.

Fluorescence intensity measurements were performed using the Mac Biophotonics ImageJ software (version 1.41n). For analysis of cell surface protein expression, cells were carefully outlined and mean fluorescence intensity of the soma was subtracted. For total protein expression analysis, somata of neurons were carefully outlined and the mean intensity of the fluorescence signals was measured. An area of each image containing no specific signals was selected for determining background staining and was subtracted from the image.

Colocalization studies were performed using Imaris (version 7.1.1, Bitplane, Zurich, Switzerland). Images were smoothed using the median filter tool (filter size: 3x3x1) and further processed by setting threshold cutoffs for each channel to exclude background staining. Colocalization channels were built (colocalization intensity 255, constant value) and protein clusters (>15 pixels) as well as colocalized clusters were counted within a randomly selected 30 μ m² area of the somata.

Gene expression assays – GABA_{B1}, GABA_{B2} and CHOP RNA levels were determined in cortical primary neurons using real-time PCR. Total RNA was extracted from neurons using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich) according to the manufactures recommendations. Reverse transcription was performed with the QuantiTec Reverse Transcription Kit (Quiagen). Quantitative real-time PCR (7900HT Fast Real-Time PCR System; Applied Biosciences) was done using the prepared cDNA and TaqMan Gene Expression Assays (Applied Biosciences) for GABA_{B1} (Gabbr1, Assay ID Rn00578911_m1), GABA_{B2} (Gabbr2, Assay ID Rn00582550_m1), CHOP (Ddit3, Assay ID Rn00492098_g1) and β -actin (Actb, Assay ID Mm00607939_s1) as a control. Quantification of RNA levels was done using the $\Delta\Delta$ Ct method.

Baclofen-Induced ERK_{1/2} Phosphorylation – GABA_B receptor activity was indirectly determined by measuring the levels of baclofen-induced ERK_{1/2} phosphorylation (28-31). Cortical neurons were incubated with the GABA_B receptor agonist baclofen for 10 min at 37 °C, 5% CO₂ or were left untreated for controls. Subsequently, the cultures were placed on ice, fixed with 4% paraformaldehyde for 15 min at 4°C and permeabilized with 0.2 % Triton X-100 for 6 min. For determination of ERK_{1/2} phosphorylation, cultures were incubated overnight at 4°C with antibodies directed against total ERK_{1/2} as well as with antibodies against diphosphorylated ERK_{1/2}. After washing with PBS, secondary antibodies were added for 1h at room temperature and neurons were analyzed by measuring fluorescence intensities of total and diphosphorylated ERK_{1/2} levels using confocal laser scanning microscopy. Levels of phosphorylated ERK_{1/2} were normalized to total ERK_{1/2} levels. Specificity of the baclofen-induced ERK_{1/2} phosphorylation was determined using the GABA_B receptor antagonist CPG 56999A.

In situ proximity ligation assay (in situ PLA) – The *in situ* PLA is a highly sensitive antibody-based method for the visualization of protein-protein interactions and posttranslational modification in cultured cells and tissue section (32,33). This method employs two primary antibodies detecting the proteins of interest raised in different species and corresponding secondary antibodies (PLA probes) tagged with oligonucleotides. Only when the proteins of interest are in close proximity (<30 nm) specific connector oligonucleotides can be hybridized and ligated to the oligonucleotides attached to the secondary antibodies forming a circular oligonucleotide. Rolling circle amplification then creates a large DNA strand to which numerous fluorophor-labeled oligonucleotides (detection probes) are hybridized. This generates a bright fluorescent spot that can be easily detected by microscopy. Quantification is done by counting the number of spots.

Here we used *in situ* PLA for analyzing the interaction of CHOP with GABA_B receptors and the heterodimerization of GABA_{B1} and GABA_{B2}. *In situ* PLA was performed using the Duolink Kit (Olink Bioscience) according the manufacturers protocol as described previously (34). The

specificity of the PLA signal was verified for both pairs of antibodies in HEK 293 cells expressing or not expressing one of the interaction partners. Furthermore, leaving out one of the primary antibodies completely prevented PLA signals. For *in situ* PLA neurons were fixed and permeabilized as described above and incubated with primary antibodies (in PBS/10% NGS) overnight at 4°C. After *in situ* PLA, neurons were analyzed by confocal laser scanning microscopy as described above. Protein-protein interactions were quantified by counting signal dots using the ImageJ software. Image-stacks (5 optical sections spaced by 0.4 µm) of individual neurons were merged visualizing maximum intensities and number of maxima per area was determined.

Forward trafficking assay – For visualization of the amount of GABA_B receptors inserted into the plasma membrane within a time period of 16 h, neurons were incubated in culture medium with antibodies directed against GABA_{B2} for 2 h at 37°C. Following washes with ACSF to remove unbound primary antibody, cells were incubated for 2 h with a large excess of Alexa 488 conjugated secondary antibody to label (i. e. mask) the existing pool of cell surface receptors. After washing, the neurons were further incubated in culture medium for 16 h at 37°C to allow neosynthesis of GABA_B receptors and forward trafficking of receptors to the plasma membrane. Neurons were then placed on ice and stained at 4°C for receptors newly inserted into the plasma membrane using antibodies directed against GABA_{B2} and Cy3-conjugated secondary antibodies. Neurons were then processed for confocal laser scanning microscopy. Controls for judging the efficiency of labeling (i. e. masking) the pool of cell surface receptors were treated in exactly the same but kept at 4°C for the 16 h incubation period.

Electrophysiology – Neurons of thapsigargin-treated or untreated control cultures were recorded in the whole-cell voltage-clamp configuration at room temperature. Spontaneously occurring postsynaptic currents (sPSCs) were recorded before, during and after the application of 50 µM baclofen at a holding potential of -60 mV. Patch electrodes were pulled from borosilicate glass and were filled with 120 mM CsCl, 10 mM EGTA, 10 mM HEPES (pH 7.4), 4 mM MgCl₂, 0.5 mM GTP and 2 mM ATP. The external solution contained

140 mM NaCl, 10 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES pH 7.4 and 10 mM glucose. Recordings were performed with a HEKA EPC-7 amplifier and Patch Master v2.11 software (HEKA Elektronik, Germany). Baclofen (50 μ M) was locally applied using an outlet tube (200 μ m ID) of a custom-designed gravity-fed micro-perfusion system positioned 50-100 μ m of the recorded neuron. All the synaptic events displaying amplitudes above the background noise (5–12 pA) were identified and analyzed off-line using the MiniAnalysis 6.0.7 software (Synaptosoft). Mean amplitudes and frequency values were obtained from 1 min-long recordings for each experimental condition and normalized.

Oxygen glucose deprivation (OGD) model of ischemia – Primary cortical neurons were washed twice with DMEM without glucose or with DMEM containing glucose for controls. For OGD, neurons were incubated for 10 min at 37°C in 95% N₂/5% CO₂ pre-bubbled glucose-free DMEM in an airtight box filled with 95% N₂/5% CO₂. Control cultures were incubated in DMEM containing glucose at 37°C in a physiological oxygen-containing environment. Subsequently, cells were washed twice with DMEM containing glucose and incubated for 24 h in their original culture medium.

RESULTS

CHOP Interacts with GABA_B Receptors and Downregulates Cell Surface Receptors in Neurons

– We had previously shown that the stress-induced transcription factor CHOP, which is only marginally expressed under normal physiological condition, interacts with GABA_B receptors by binding to the coiled-coil motif in the C-terminal domain of GABA_{B2} and with a so far unidentified site in GABA_{B1} (22). Upon overexpression in HEK 293 cells, interaction of CHOP with GABA_B receptors resulted in the intracellular accumulation and a reduced cell surface expression of the receptors by an as yet unknown mechanism (22). To confirm an interaction of GABA_B receptors with CHOP in neurons, we overexpressed CHOP in cultured cortical neurons and tested for an interaction with GABA_B receptors using the *in situ* proximity ligation assay (*in situ* PLA, (32,33)). In non-transfected neurons only very few PLA dots were detected, being in line with a low expression level of CHOP under normal physiological

conditions. However, overexpression of CHOP dramatically increased PLA signals, indicating numerous GABA_B receptor-CHOP interactions (Fig. 1A).

Because ER stress-induced up-regulation of CHOP has been shown to play an important role in ischemia-induced neuronal death (35-42) we then analyzed the mechanism of CHOP-induced down-regulation of cell surface GABA_B receptors by exposing cultured cortical neurons to ER stress. ER stress was induced by treating neurons for 2 h with the sarco/endoplasmic reticulum Ca²⁺-ATPase blocker thapsigargin. After a 16 h recovery period, neurons were analyzed for CHOP and GABA_B receptor interaction using *in situ* PLA. Under control conditions, i. e. in untreated neurons, only few interactions were observed (Fig. 1B). However, upon up-regulation of CHOP with thapsigargin, numerous interactions with GABA_B receptors were detected ($454 \pm 53\%$ of control, $n=30$, $p < 0.001$, Fig. 1B). Thus, up-regulation of CHOP by ER stress resulted in an interaction with GABA_B receptors. Under these conditions, no *in situ* PLA signals were generated using CHOP and GABA_A receptor antibodies, documenting the specificity of the CHOP-GABA_B receptor interaction (Fig. 1C).

An analysis of the protein expression levels revealed that CHOP was vastly up-regulated in thapsigargin-treated neurons ($706 \pm 65\%$ of control, $n=57$, $p < 0.001$; Fig. 2A), whereas the cell surface staining of GABA_{B1} and GABA_{B2} was significantly decreased (GABA_{B1}: $67 \pm 4\%$, GABA_{B2}: $50 \pm 3\%$ of control, $n=50-55$, $p < 0.001$; Fig. 2A). No colocalization of CHOP with cell surface GABA_B receptors was detected. In contrast to cell surface receptors, total GABA_{B1} and GABA_{B2} expression levels remained unchanged in thapsigargin-treated neurons (Fig. 2B). These findings suggest that the interaction of CHOP with GABA_B receptors caused a down-regulation of the receptors from the cell surface and their intracellular accumulation.

To verify that the loss of cell surface receptors was caused by the interaction of CHOP with GABA_B receptors, neurons were transfected with mutant forms of CHOP that are unable to interact with GABA_{B1} (CHOP Δ N, N-terminal deletion (22)) or GABA_{B2} (CHOP Δ LZ, deletion of C-terminal leucine zipper motif (22)). Neurons overexpressing wild type CHOP displayed a

significantly reduced level of cell surface GABA_{B2} compared to control neurons transfected with EGFP ($63 \pm 4\%$, $n=82$, $p < 0.001$; Fig. 2C). However, overexpressing either of the CHOP mutants did not affect cell surface expression of GABA_B receptors (CHOP Δ LZ: $92 \pm 5\%$, $p > 0.05$, $n=79$; CHOP Δ N: $103 \pm 5\%$, $n=101$, $p > 0.05$), suggesting that down-regulation of cell surface receptors was based on its interaction with CHOP.

To rule out that up-regulation of CHOP affects transcription of GABA_{B1} and GABA_{B2}, their mRNA levels were quantified in neurons treated with thapsigargin using real-time PCR (Fig. 2D). CHOP mRNA levels were drastically increased in thapsigargin-treated neurons ($432 \pm 54\%$ of control, $n=4$, $p < 0.001$) but GABA_{B1} and GABA_{B2} mRNA levels remained unchanged (GABA_{B1}: $113 \pm 6\%$ of control, $n=5$, $p > 0.05$; GABA_{B2}: $94 \pm 12\%$ of control, $n=5$, $p > 0.05$). Thus, CHOP does not regulate GABA_B receptor expression at the transcriptional level.

CHOP-Mediated Down-regulation of Cell Surface Receptors Reduces GABA_B Receptor Signaling – It is well established that GABA_B receptors activate extracellular-signal-regulated kinase 1/2 (ERK_{1/2}) in neurons (28-31). To analyze the functional consequences of CHOP-mediated down-regulation of GABA_B receptors, receptor activity was assayed based on baclofen-induced ERK1/2 phosphorylation. Activation of GABA_B receptors with 100 μ M baclofen in control neurons significantly increased ERK_{1/2} phosphorylation ($159 \pm 10\%$, $n=26$, $p < 0.001$; Fig. 3A), which was abolished by preincubation of neurons with the GABA_B receptor antagonist CPG 56999A ($106 \pm 10\%$, $n=26$, $p > 0.05$; Fig. 3A). However, in thapsigargin-treated neurons no increase of ERK_{1/2} phosphorylation was observed ($108 \pm 8\%$ of control, $n=28$, $p > 0.05$; Fig. 3A), indicating that CHOP-mediated down-regulation of cell surface GABA_B receptors inhibits downstream signaling.

To test whether CHOP-induced down-regulation of GABA_B receptors affects GABA_B receptor-mediated neuronal inhibition, spontaneous synaptic activity was measured in electrophysiological experiments using the whole-cell voltage-clamp configuration. In untreated control neurons, application of baclofen considerably decreased the amplitude ($66 \pm 6\%$ of control, $n=9$) and the frequency ($38 \pm 8\%$ of

control, $n=5$) of spontaneous postsynaptic currents (sPSCs) (Fig. 3B). However, after up-regulation of CHOP by treating cultures with thapsigargin, baclofen-induced inhibition of sPSCs was strongly reduced (amplitude: $87 \pm 6\%$ of control $n=9$, $p < 0.05$, frequency: $65 \pm 9\%$ of control, $n=5$, $p < 0.05$; Fig. 3B). This finding is consistent with an up-regulation of CHOP and subsequently reduced levels of functional GABA_B receptors available for neuronal inhibition.

CHOP Interacts with GABA_B Receptors in the ER and Interferes with Receptor Heterodimerization – Next, we investigated the mechanism of CHOP-mediated down-regulation of cell surface GABA_B receptors. We envisioned two scenarios: 1) CHOP disrupts functional GABA_B receptor heterodimers on the cell surface, which would lead to their internalization and degradation, or 2) CHOP interacts with GABA_B receptors in the ER, inhibits receptor heterodimerization and consequently forward transport to the cell surface. To test these two scenarios, we analyzed the colocalization of CHOP and GABA_B receptor subunits in different cellular compartments in untreated control neurons and neurons treated with thapsigargin to upregulate CHOP. In thapsigargin-treated neurons, GABA_B receptors accumulated in the ER as indicated by an increased colocalization of GABA_{B1} (control: 10 ± 0.7 clusters, $n=20$, thapsigargin: 15 ± 0.8 clusters, $n=21$, $p < 0.001$) and GABA_{B2} (control: 7 ± 0.7 clusters, $n=32$, thapsigargin: 13 ± 0.7 clusters, $n=32$, $p < 0.001$) with the ER marker protein disulfide isomerase (PDI, Fig. 4A, B). Hardly any CHOP was observed in the ER under control conditions, whereas CHOP accumulated in the ER after thapsigargin treatment (GABA_{B1} stained neurons, control: 2 ± 0.4 clusters, $n=20$, thapsigargin: 9 ± 0.9 clusters, $n=21$, $p < 0.001$; Fig. 4A; GABA_{B2} stained neurons, control: 1.5 ± 0.4 clusters, $n=32$, thapsigargin: 9 ± 0.6 clusters, $n=32$, $p < 0.001$; Fig. 4B). Triple-colocalization of GABA_B-CHOP-PDI was basically absent under control conditions but significantly increased after up-regulation of CHOP by thapsigargin (Fig. 4A, B; GABA_{B1}-CHOP-PDI, control: 1.4 ± 0.3 clusters, $n=20$, thapsigargin: 6 ± 0.7 clusters, $n=21$, $p < 0.01$; GABA_{B2}-CHOP-PDI, control: 0.7 ± 0.3 clusters, $n=32$, thapsigargin: 6 ± 0.5 clusters, $n=32$, $p < 0.001$). These findings indicate that GABA_B receptors interact with CHOP in the ER, which

leads to the accumulation of GABA_{B1} and GABA_{B2} in the ER.

In contrast, GABA_B receptors did not display increased colocalization with the Golgi apparatus marker (GM) GM130 in thapsigargin-treated neurons (control: 27 ± 3 clusters, $n=19$, thapsigargin: 30 ± 3 clusters, $p > 0.05$, $n=19$; Fig. 4C), indicating no accumulation of GABA_B receptors in the Golgi after up-regulation of CHOP. In line with this finding, there was no statistically significant increase in CHOP-GM colocalization control: 3 ± 1 clusters, $n=19$; thapsigargin: 7 ± 2 clusters, $p > 0.05$, $n=19$, $p > 0.05$; Fig. 4C) nor in GABA_{B2}-CHOP-GM (control: 2 ± 0.7 clusters, $n=19$, thapsigargin: 6 ± 2 clusters, $n=19$, $p > 0.05$; Fig. 4C), indicating marginal interactions between CHOP and GABA_B receptors in the Golgi after thapsigargin-induced up-regulation of CHOP.

In addition, there was no significant colocalization of CHOP with GABA_B receptors at the cell surface (control: 1.3 ± 0.2 clusters, $n=45$; thapsigargin: 2.3 ± 0.5 clusters, $n=44$, $p > 0.05$; Fig. 4D), indicating that GABA_B receptors do not interact with CHOP in the plasma membrane.

To demonstrate that the accumulation of GABA_B receptors in the ER is mediated by direct interaction with CHOP, we expressed in neurons a mutant form of GABA_{B2} lacking its leucine zipper (GABA_{B2}ΔLZ), and thus its CHOP interaction site, together with GABA_{B1} containing a mutated ER retention signal (GABA_{B1}ASA) to ensure forward trafficking of the receptors. GABA_{B1}ASA was required because deletion of the GABA_{B2} leucine zipper unmasks the ER retention signal in the C-terminus of GABA_{B1} and therefore prevents forward trafficking of the receptors. To verify normal trafficking behavior of the GABA_{B1}ASA/GABA_{B2}ΔLZ heterodimers we analyzed the colocalization of GABA_{B1}ASA and GABA_{B2}ΔLZ with PDI and CHOP in thapsigargin-treated and untreated neurons. In thapsigargin-treated neurons, GABA_{B1}ASA (control: 10 ± 3 clusters, $n=12$; thapsigargin: 15 ± 5 clusters, $n=19$, $p < 0.01$, Fig. 5A), GABA_{B2} (control: 13 ± 5 clusters, $n=17$; thapsigargin: 17 ± 4 clusters, $n=15$, $p < 0.01$, Fig. 5B) and CHOP (Fig. 5A, control: 1.3 ± 1.4 clusters, $n=13$, thapsigargin: 11 ± 5 clusters, $n=20$, $p < 0.001$; Fig. 5B, control: 1.4 ± 1.8 clusters, $n=17$, thapsigargin: 11 ± 5 clusters, $n=16$, $p < 0.001$) showed increased colocalization with the ER marker PDI. Also, increased colocalization

of GABA_{B1} or GABA_{B2} with CHOP and PDI (GABA_{B1}/CHOP/PDI and GABA_{B2}/CHOP/PDI) was detected in thapsigargin-treated neurons (GABA_{B1}ASA, control: 0.7 ± 1.1 clusters, $n=13$, thapsigargin: 7 ± 4 clusters, $n=20$, $p < 0.001$; GABA_{B2}, control: 0.8 ± 1.1 clusters, $n=17$, thapsigargin: 6 ± 3 clusters, $n=16$, $p < 0.001$; Fig. 5A, B). The identical colocalization patterns of GABA_{B1}ASA/GABA_{B2} receptors and endogenous GABA_B receptors indicate normal trafficking capabilities of the mutant receptors.

However, transfection of neurons with GABA_{B1}ASA/GABA_{B2}ΔLZ receptors, which lack the CHOP interaction site in GABA_{B2}, fully abolished the increased colocalization of GABA_{B1}-PDI (control: 13 ± 4 clusters, $n=14$; thapsigargin: 10 ± 4 clusters, $n=20$, $p > 0.05$), GABA_{B2}-PDI (control: 12 ± 5 clusters, $n=14$; thapsigargin: 9 ± 3 clusters, $n=11$, $p > 0.05$) as well as GABA_{B1}-CHOP-PDI (control: 0.5 ± 1.1 clusters, $n=15$; thapsigargin: 1.5 ± 1.5 clusters, $n=22$, $p > 0.05$) and GABA_{B2}-CHOP-PDI (control: 0.3 ± 0.8 clusters, $n=14$; thapsigargin: 1.3 ± 1.2 clusters, $n=12$, $p > 0.05$; Fig. 5A, B) after induction of CHOP with thapsigargin. This result verified that CHOP directly interacts with GABA_B receptors to induce their accumulation in the ER.

Because GABA_{B1} and GABA_{B2} interact via the leucine zippers in their C-terminal domains, binding of CHOP to GABA_{B2} may prevent GABA_B receptor heterodimerization and thus inhibit forward trafficking of the receptors. Therefore, we tested whether up-regulation of CHOP interferes with heterodimerization of GABA_{B1} and GABA_{B2} using *in situ* PLA. In thapsigargin-treated neurons we observed a significant reduction of *in situ* PLA signals ($64 \pm 5\%$ of control, $n=32$, $p < 0.001$; Fig. 5C), indicating the presence of considerably less GABA_{B1}/GABA_{B2} heterodimers as compared to untreated control neurons. This result suggests that interaction of CHOP with GABA_B receptors disrupts or prevents heterodimerization of GABA_{B1} and GABA_{B2}.

These findings suggest a mechanism, in which CHOP interacts with GABA_B receptors in the ER after cellular stress to prevent heterodimerization of GABA_{B1} and GABA_{B2}. Because only heterodimerized GABA_B receptors can leave the ER (2-4), this mechanism is expected to impede

forward trafficking of newly synthesized receptors to the plasma membrane.

Up-regulation of CHOP Prevents Forward Trafficking of GABA_B Receptors to the Cell Surface – To test whether up-regulation of CHOP impairs forward trafficking of GABA_B receptors to the cell surface we transfected neurons with HA-tagged GABA_{B2} and CHOP. Twenty-four hours after transfection newly synthesized HA-tagged receptors inserted into the plasma membrane were detected using an anti-HA antibody. In control neurons only transfected with HA-GABA_{B2} strong cell surface expression of HA-tagged GABA_{B2} was detected (Fig. 6A). However, in neurons transfected with CHOP staining for cell surface HA-GABA_{B2} was strongly reduced ($36 \pm 4\%$ of control, $n=25$, $p < 0.001$; Fig. 6A). This reduction of cell surface HA-GABA_{B2} was not observed in neurons expressing a mutant of CHOP (CHOP Δ LZ) that is unable to bind to GABA_{B2} ($80 \pm 7\%$ of control, $n=25$, $p > 0.05$; Fig. 6A). These results suggest that the interaction of CHOP with GABA_{B2} impairs forward trafficking of GABA_B receptors to the cell surface.

This result was confirmed for native GABA_B receptors with an immunofluorescence-based forward trafficking assay using untreated control neurons and thapsigargin-treated neurons. After masking the existing receptor pool on the cell surface with primary and secondary antibodies, the insertion of new receptors into the plasma membrane was tested after 16 h. Membrane insertion of GABA_B receptors was drastically reduced in neurons expressing high levels of CHOP ($16 \pm 6\%$ of control, $n=33$, $p < 0.001$; Fig. 6B).

Down-regulation of Cell Surface GABA_B Receptors by CHOP in the OGD Model of Ischemia – Next we tested whether up-regulation of CHOP under ischemic conditions mediates down-regulation of cell surface GABA_B receptors. We used the oxygen and glucose deprivation (OGD) *in vitro* model of ischemia, in which cultured cortical neurons were deprived for 10 min from oxygen and glucose followed by a recovery period of 24 h. 24 h after OGD, CHOP expression in neurons was significantly increased ($157 \pm 5\%$ of control, $n=311$, $p < 0.001$; Fig. 7A). As expected, cell surface GABA_B receptors considerably decreased 24 h after OGD in CHOP expressing

neurons ($44 \pm 3\%$ of control, $n=49$, $p < 0.001$; Fig. 7B).

To test whether the interaction of CHOP with GABA_B receptors is responsible for the down-regulation of cell surface receptors after OGD, we overexpressed in cortical neurons CHOP Δ LZ, a mutant form of CHOP that is not able to interact with GABA_{B2} and does not affect cell surface numbers of GABA_B receptors (Fig. 1C). Endogenous CHOP was significantly up-regulated after OGD in transfected ($154 \pm 8\%$ of control, $n=20$, $p < 0.001$) and in non-transfected neurons ($167 \pm 12\%$ of control, $n=22$, $p < 0.001$; Fig. 8). In non-transfected neurons, GABA_{B2} receptors were down-regulated after OGD ($60 \pm 7\%$ of control, $n=27$, $p < 0.001$; Fig. 8), whereas in neurons overexpressing CHOP Δ LZ, despite similar up-regulation of endogenous CHOP, the down-regulation of GABA_B receptors was completely prevented ($113 \pm 7\%$ of control, $n=45$, $p > 0.05$; Fig. 8). These results verify that down-regulation of cell surface GABA_B receptors after ischemic conditions is mediated by their interaction with CHOP.

DISCUSSION

GABA_B receptor-mediated neuronal inhibition critically depends on the availability of receptors in the plasma membrane. Receptor numbers might be altered under pathological conditions and a loss of receptors resulting in diminished neuronal inhibition is expected to contribute to the disease state. We previously showed that the ER stress-induced transcription factor CHOP interacts with GABA_B receptors, causing their down-regulation from the cell surface upon coexpression in HEK 293 (22). This finding suggests that CHOP, besides its function as transcription factor, may regulate GABA_B receptor-mediated neuronal inhibition by affecting the availability of cell surface receptors. Because CHOP is expressed at very low levels under normal physiological conditions but is highly up-regulated after induction of ER-stress (21,43-45), CHOP-induced down-regulation of cell surface GABA_B receptors may be a contributing factor to neurological disease states associated with ER stress, such as stroke, Alzheimer's and Parkinson's disease or bipolar disorders (20).

In the present study, we verified that ER stress-induced CHOP expression down-regulates cell

surface GABA_B receptors also in neurons and disclosed the mechanism underlying this effect. An *in vitro* model of ischemia suggests that this mechanism is operative in cerebral ischemia.

We induced up-regulation of CHOP in cultured neurons by inhibition of the sarco/endoplasmic reticulum Ca²⁺-ATPase with thapsigargin, which leads to the depletion of Ca²⁺ from the ER thereby causing ER stress (46). Thapsigargin up-regulated CHOP in neurons and considerably down-regulated GABA_B receptors from the cell surface. The reduced level of cell surface receptors consequently affected downstream signaling of GABA_B receptors as shown by impaired ERK_{1,2} phosphorylation and reduced baclofen-induced inhibition of spontaneous neuronal activity.

Down-regulation of cell surface GABA_B receptors was mediated by the interaction of the receptors with CHOP since overexpression in neurons of mutant forms either lacking the site binding to GABA_{B1} or the site binding to GABA_{B2} prevented down-regulation of the receptors. Most importantly, up-regulation of CHOP did not affect the mRNA levels of GABA_{B1} and GABA_{B2}, ruling out a contribution of impaired subunit transcription (Fig. 2D).

Up-regulated CHOP selectively accumulates together with GABA_B receptors in the ER, suggesting that interaction with CHOP retains GABA_B receptors in the ER and prevents their forward trafficking to the cell surface. This conclusion is supported by the finding that mutant GABA_B receptors not containing the CHOP binding site in GABA_{B2} do not accumulate in the ER and that the insertion of new receptors into the plasma membrane is strongly reduced upon up-regulation of CHOP. The mechanism that interferes with forward trafficking of the receptors appears to involve prevention or disruption of receptor heterodimerization as indicated by our *in situ* PLA experiments. It is currently not clear whether CHOP prevents heterodimerization of newly synthesized GABA_{B1} and GABA_{B2} by binding to GABA_{B1} and GABA_{B2} or directly disrupts already existing heterodimers. In either case, preventing heterodimerization exposes the ER retention signal in the C-terminal domain of GABA_{B1}, which prohibits ER exit of GABA_{B1} (2-4). In addition, GABA_{B2} contains a C-terminal sequence (amino acids 841-862) which is important for forward trafficking (47). Binding of

CHOP to the leucine-zipper domain of GABA_{B2}, upstream of this motif, might sterically interfere with the function of this motif and prevent ER export of GABA_{B2}.

The mechanism of CHOP-induced down-regulation of cell surface receptors appears to be operative under ischemic conditions. This is not surprising because cerebral ischemia has been shown to be associated with ER stress and the profound up-regulation of CHOP (35-42). Using the OGD *in vitro* model of ischemia, we found that up-regulated CHOP mediates down-regulation cell surface GABA_B receptors, which depended on the interaction of CHOP with the receptors.

So far, the effect of cerebral ischemia on GABA_B receptor expression levels has been rarely investigated and the results are difficult to correlate because different animal species and experimental conditions were used. *In vivo* models of ischemia demonstrated a loss of GABA_B receptors 1-4 days after the insult (7-9). Because considerable ischemia-induced neuronal death occurs during this time period, the loss of GABA_B receptors might be due, at least in part, to a loss of GABA_B receptor expressing neurons. A recent *in vitro* study on cultured hippocampal slices using a similar OGD paradigm as in this study detected down-regulation of total GABA_{B2} but no effect on the expression of total GABA_{B1} (10). In the present study, we detected down-regulation of both GABA_{B1} and GABA_{B2} selectively from the cell surface but did not observe changes in total receptor expression. The reason for this discrepancy is not clear yet but it may be caused by different experimental conditions used (organotypic hippocampal slices, 45 min OGD versus cultured cortical neurons, 10 min OGD).

Cerebral ischemia is associated with excessive glutamate release and overstimulation of glutamate receptors triggers a signaling cascade leading to neuronal death (48). The enhanced neuronal activity also increases extracellular levels of GABA (6), which in turn should activate GABA_B receptors located at glutamatergic synapses to counteract, at least partially, the increased neuronal excitation to reduce excitotoxicity. The down-regulation of functional GABA_B receptors from the cell surface might therefore be one factor that fosters excitotoxicity. It is well established that sustained activation of GABA_B receptors (by application of baclofen) under ischemic conditions

reduces neuronal cell death (10-19). In this regard, it is conceivable that restoring normal cell surface expression of GABA_B receptors under ischemic conditions would also reduce excitotoxicity. This view is supported by a recent study showing that mild hypothermia reverses down-regulation of total GABA_{B1} after cerebral ischemia by an unknown mechanism and reduces neuronal death (8).

One opportunity to restore normal cell surface GABA_B receptor levels would be the application of small synthetic peptides interfering with the CHOP-GABA_B receptor interaction. Further experiments are required to identify small peptide sequences that inhibit the interaction of CHOP

with GABA_B receptors but do not interfere with receptor heterodimerization. This approach provides the opportunity to unambiguously test the hypothesis whether CHOP-induced down-regulation of cell surface GABA_B receptors promotes excitotoxicity. If this is the case small interfering peptides may be of potential therapeutic use to limit neuronal death under ischemic conditions. Such an intervention would be highly specific because CHOP is significantly expressed only upon ER stress and it targets a specific protein-protein interaction in response to the ischemic insult and is thus expected to be associated with low risk of side effects.

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FOOTNOTES

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The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; GABA, γ -aminobutyric acid; OGD, oxygen and glucose deprivation; PLA, proximity ligation assay; sPSCs, spontaneous postsynaptic currents.

FIGURE LEGENDS

Figure 1. Up-regulated CHOP interacts with GABA_B receptors in neurons.

A, CHOP over-expressed in neurons interact with GABA_B receptors. Cortical neurons were left non-transfected (-CHOP) or were transfected with CHOP (+CHOP) and analyzed for interaction with GABA_B receptors by *in situ* PLA using antibodies directed against CHOP and GABA_{B2}. Because under normal physiological conditions CHOP expression is low, only few interactions (white dots in representative images) were detected. However, overexpression of CHOP resulted in numerous CHOP-GABA_B receptor interactions. The images represent merged Z-stack reconstructions of 5 optical section spaced by 0.4 μ m. Scale bar: 5 μ m.

B, Thapsigargin-induced CHOP interacts with GABA_B receptors. Cortical neurons were incubated with 2 μ M thapsigargin for 2 h and were allowed to recover in their original culture medium for 16 h. The interaction of CHOP with GABA_B receptors was determined by *in situ* PLA using antibodies directed against CHOP and GABA_{B2} (white dots in representative images, left; scale bar: 5 μ m). Right: quantification of *in situ* PLA signals. Means \pm S.E.M., 30 neurons from four experiments. ***, $p < 0.001$, t-test.

C, CHOP does not interact with GABA_A receptors. Cortical neurons were treated (TG) or not (Ctrl) with 2 μ g thapsigargin for 2 h and were allowed to recover in their original culture medium for 16 h. The interaction of CHOP with GABA_A receptors was determined by *in situ* PLA using antibodies directed against CHOP and the $\alpha 1$ subunit of GABA_A receptors. No interactions were observed (right panels). As a positive control, the interaction of the GABA_A receptor subunits $\alpha 1$ and $\beta 2/3$ was tested. Numerous interactions were detected in untreated neurons (white dots in representative image, left panel). The images represent merged Z-stack reconstructions of 5 optical section spaced by 0.4 μ m. Scale bar: 5 μ m.

Figure 2. Up-regulated CHOP in neurons mediate down-regulation of cell surface GABA_B receptors.

A, Up-regulation of CHOP down-regulates cell surface GABA_B receptors. Neurons were treated with thapsigargin and stained for surface GABA_{B1} and GABA_{B2} (green). Subsequently cells were fixed, permeabilized and stained for CHOP (red). Neurons not treated with thapsigargin served as controls. Left: representative images; scale bar: 7 μ m. Right: quantification of cell surface GABA_{B1}, GABA_{B2} and CHOP. Means \pm S.E.M., 50-55 neurons from five experiments. ***, $p < 0.001$, t-test.

A, Up-regulation of CHOP does not affect total GABA_B receptor expression levels. Neurons were treated with thapsigargin and stained for total GABA_{B1} and GABA_{B2} (green) and CHOP (red). Neurons not treated with thapsigargin served as controls (Ctrl). Scale bar: 10 μ m. Bar graphs show quantification of

total expression levels of GABA_{B1} and GABA_{B2}. Means \pm S.E.M., 30 neurons from three experiments. n.s., $p > 0.05$, t-test.

C, Mutant forms of CHOP that cannot bind to GABA_B receptors do not mediate down-regulation of cell surface receptors. Neurons were cotransfected with EGFP and CHOP or mutant forms of CHOP lacking its interaction site with GABA_{B2} (CHOP Δ LZ) or its interaction site with GABA_{B1} (CHOP Δ N). After 48 h, transfected neurons were stained for cell surface GABA_{B2} (green) and for CHOP (red). Left: representative images; scale bar: 10 μ m. Right: quantification of cell surface GABA_{B2} levels in neurons expressing either CHOP, CHOP Δ LZ or CHOP Δ N. Means \pm S.E.M., 82 (EGFP and CHOP), 79 (CHOP Δ LZ) and 101 (CHOP Δ N) neurons from three experiments. ***, $p < 0.001$, one way ANOVA, Bonferroni's multiple comparison test.

D, CHOP does not regulate GABA_B receptor mRNA levels. Total mRNA was isolated from neuronal cultures treated or not treated (controls) with thapsigargin. CHOP, GABA_{B1} and GABA_{B2} mRNA was quantified using real-time PCR. Means \pm S.E.M., 4 (CHOP) and 5 (GABA_{B1} and GABA_{B2}) individual cultures were analyzed. ***, $p < 0.001$, n.s.: not significant ($p > 0.05$), t-test.

Figure 3. CHOP-mediated down-regulation of cell surface GABA_B receptors impairs GABA_B receptor-mediated downstream signaling.

A, GABA_B receptor-induced phosphorylation of ERK_{1/2} was prevented by the GABA_B receptor antagonist CPG 56999A and by thapsigargin-induced up-regulation of CHOP. Neurons were treated with thapsigargin (+TG) to upregulate CHOP while control cultures remained untreated. Subsequently, neurons were incubated for 10 min with 100 μ M baclofen in the presence or absence of 10 μ M CPG 56999A (+CGP, GABA_B receptor antagonist). After fixation and permeabilization neurons were stained for total ERK_{1/2} (ERK, green) and diphosphorylated ERK_{1/2} (pERK, red). Left: representative images; scale bar: 5 μ m. Right: quantification of pERK levels. Means \pm S.E.M., 26 (baclofen, baclofen + CPG) and 28 (baclofen + TG) neurons from three experiments. ***, $p < 0.001$, one way ANOVA, Bonferroni's multiple comparison test.

B, Up-regulation of CHOP diminished baclofen-induced inhibition of spontaneous postsynaptic currents (sPSCs). Left: Representative current traces depicting spontaneous sPSCs recorded from untreated control neurons (n=9) or from neurons treated with thapsigargin for up-regulation of CHOP (n=5). Right: Normalized amplitude and frequency values of the sPSCs. Mean amplitudes and frequency values were normalized to the control condition of the individual neuron. Con.: control, bac.: baclofen, TG.: thapsigargin. Mean \pm S.E.M., *, $p < 0.05$, t-test.

Figure 4. Up-regulated CHOP colocalize with GABA_B receptors in the ER and induces their intracellular accumulation.

A, B, GABA_B receptors accumulate in the ER and colocalize with CHOP. Neurons were treated with thapsigargin to upregulate CHOP and stained for GABA_{B1} (A) or GABA_{B2} (B) (green), CHOP (red) and the ER marker PDI (blue). Cultures not treated with thapsigargin served as controls. Left: representative images depicting GABA_{B1/B2}-CHOP-PDI colocalization (white dots marked with arrows, scale bar: 5 μ m). Right: quantification of colocalization. Means \pm S.D., 20-32 neurons per colocalization from three experiments. ***, $p < 0.001$, t-test.

C, GABA_B receptors do not accumulate or colocalize with CHOP in the Golgi apparatus. Neurons were treated with thapsigargin to upregulate CHOP and stained for GABA_{B2} (green), CHOP (red) and the Golgi marker GM130 (blue). Cultures not treated with thapsigargin served as controls. Left: representative images, scale bar: 5 μ m. Right: quantification of colocalization. Means \pm S.D., 19 neurons per colocalization from three experiments. n.s.: not significant ($p > 0.05$), t-test.

D, GABA_B receptors do not colocalize with CHOP on the cell surface. Neurons were treated with thapsigargin to upregulate CHOP and were then stained for cell surface GABA_{B2} (green) as well as for CHOP (red). Cultures not treated with thapsigargin served as controls. Left: representative images, scale

bar: 5 μ m. Right: quantification of colocalization. Means \pm S.D., 44 (control) and 45 (thapsigargin) neurons from four experiments. n.s.: not significant ($p>0.05$), t-test.

Figure 5. Accumulation of GABA_B receptors in the ER is mediated by interaction with CHOP.

A, B, Neurons were transfected with EGFP in combination with GABA_{B1}ASA/GABA_{B2} or GABA_{B1}ASA/GABA_{B2} Δ LZ. After 48h, neurons were treated with thapsigargin to upregulate CHOP and then stained for GABA_{B1}ASA (A) or GABA_{B2} Δ LZ (B), CHOP and the ER marker PDI. Cultures not treated with thapsigargin served as controls. Left: representative images depicting colocalization of GABA_{B1}-CHOP-PDI and GABA_{B2}-CHOP-PDI (white dots, scale bar: 5 μ m). Right: quantification of colocalization. Means \pm S.D., 11-22 neurons per colocalization from two experiments. **, $p<0.01$; ***, $p<0.001$, n.s.: not significant ($p>0.05$), t-test.

C, Up-regulation of CHOP interferes with GABA_B receptor heterodimerization. Neurons were treated with thapsigargin to upregulate CHOP and tested for GABA_{B1}/GABA_{B2} heterodimerization by *in situ* PLA using antibodies directed against GABA_{B1} and GABA_{B2} (white dots in representative images, left; scale bar: 5 μ m). Cultures not treated with thapsigargin served as controls. The images represent merged Z-stack reconstructions of 5 optical section spaced by 0.4 μ m. Right: quantification of *in situ* PLA signals. Means \pm S.E.M., 32 neurons from three experiments. ***, $p<0.001$, t-test.

Figure 6. Up-regulated CHOP impairs forward trafficking of GABA_B receptors.

A, Neurons were transfected with either EGFP and HA-tagged GABA_{B2} (control), EGFP, HA-GABA_{B2} and CHOP (Chop) or EGFP HA-GABA_{B2} and CHOP Δ LZ (CHOP Δ LZ). Twenty-four hours after transfection, GABA_B receptors newly inserted into the plasma membrane were visualized by staining with HA-antibodies. Left: representative images, scale bar: 5 μ m. Right: quantification of GABA_B receptor membrane insertion. Means \pm S.E.M., 25 neurons per condition from three experiments. ***, $p<0.001$, one way ANOVA, Dunnett's post-hoc test.

B, Neurons were treated with thapsigargin to upregulate CHOP und subjected to an immunofluorescence-based forward trafficking assay to determine the amounts of GABA_B receptors inserted into the plasma membrane within a time period of 16 h (red clusters in representative images, scale bar: 5 μ m). CHOP expression is depicted in blue. Cultures not treated with thapsigargin served as controls. Right: quantification of GABA_B receptor membrane insertion. Means \pm S.E.M., 38 (control) and 33 (thapsigargin) neurons from three experiments. ***, $p<0.001$, t-test.

Figure 7. CHOP is up-regulated and GABA_B receptors are down-regulated in the OGD model of cerebral ischemia.

A, Oxygen and glucose deprivation (OGD) in neurons upregulates CHOP. Neurons were subjected to OGD for 10 min, followed by a recovery period of 24 h. Subsequently, neurons were stained for CHOP (green) and for NeuN to visualize the neurons (red). CHOP expression in neurons is depicted in yellow. Cultures not subjected to OGD served as controls. Left: representative images, scale bar: 30 μ m. Right: quantification of CHOP expression in neurons. Means \pm S.E.M., 271 (control) and 311 (thapsigargin) neurons from three experiments. ***, $p<0.001$, t-test.

B, Cell surface GABA_B receptors are down-regulated following OGD. Neurons were subjected to OGD for 10 min, followed by a recovery period of 24 h and were then stained for cell surface GABA_{B2} (green) and CHOP (red). Cultures not subjected to OGD served as controls. Left: representative images, scale bar: 5 μ m. Right: quantification of cell surface GABA_B receptor expression. Means \pm S.E.M., 30 (control) and 49 (thapsigargin) neurons from three experiments. ***, $p<0.001$, t-test.

Figure 8. Interaction of CHOP with GABA_B receptors is responsible for OGD-induced down-regulation of cell surface receptors.

Neurons were transfected with a CHOP mutant that is not able to interact with GABA_{B2} (CHOP Δ LZ) and EGFP. After 24 h, neurons were subjected to OGD for 10 min, followed by a recovery period of 24 h.

Subsequently, neurons were stained for cell surface GABA_B receptors (blue) and endogenous CHOP (red). CHOPΔLZ overexpressing neurons were selected by means of EGFP expression. Cultures not subjected to OGD served as controls. Top: representative images, scale bar: 5 μm. Bottom: quantification of cell surface GABA_B receptors. Means ± S.E.M., 38 (control, non-transfected), 27 (OGD, non-transfected), 36 (control, transfected) and 45 (OGD, transfected) neurons from three experiments. **, p<0.01, n.s.: not significant (p>0.05), t-test.

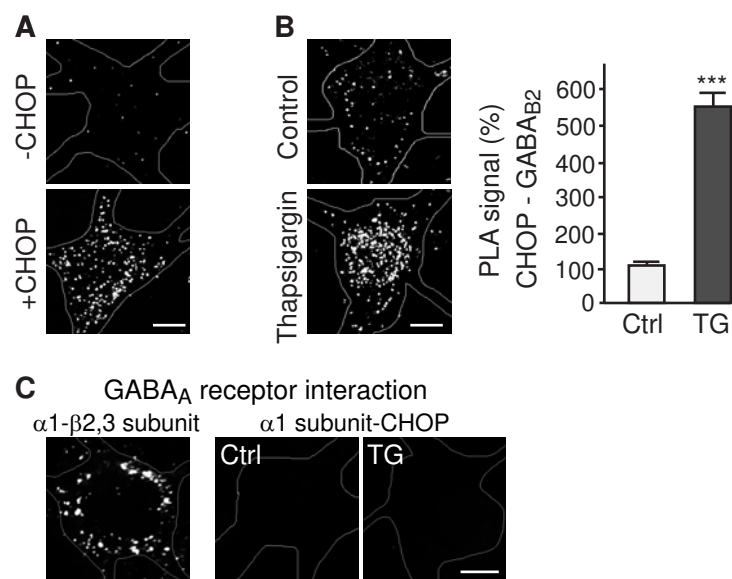


Fig. 1

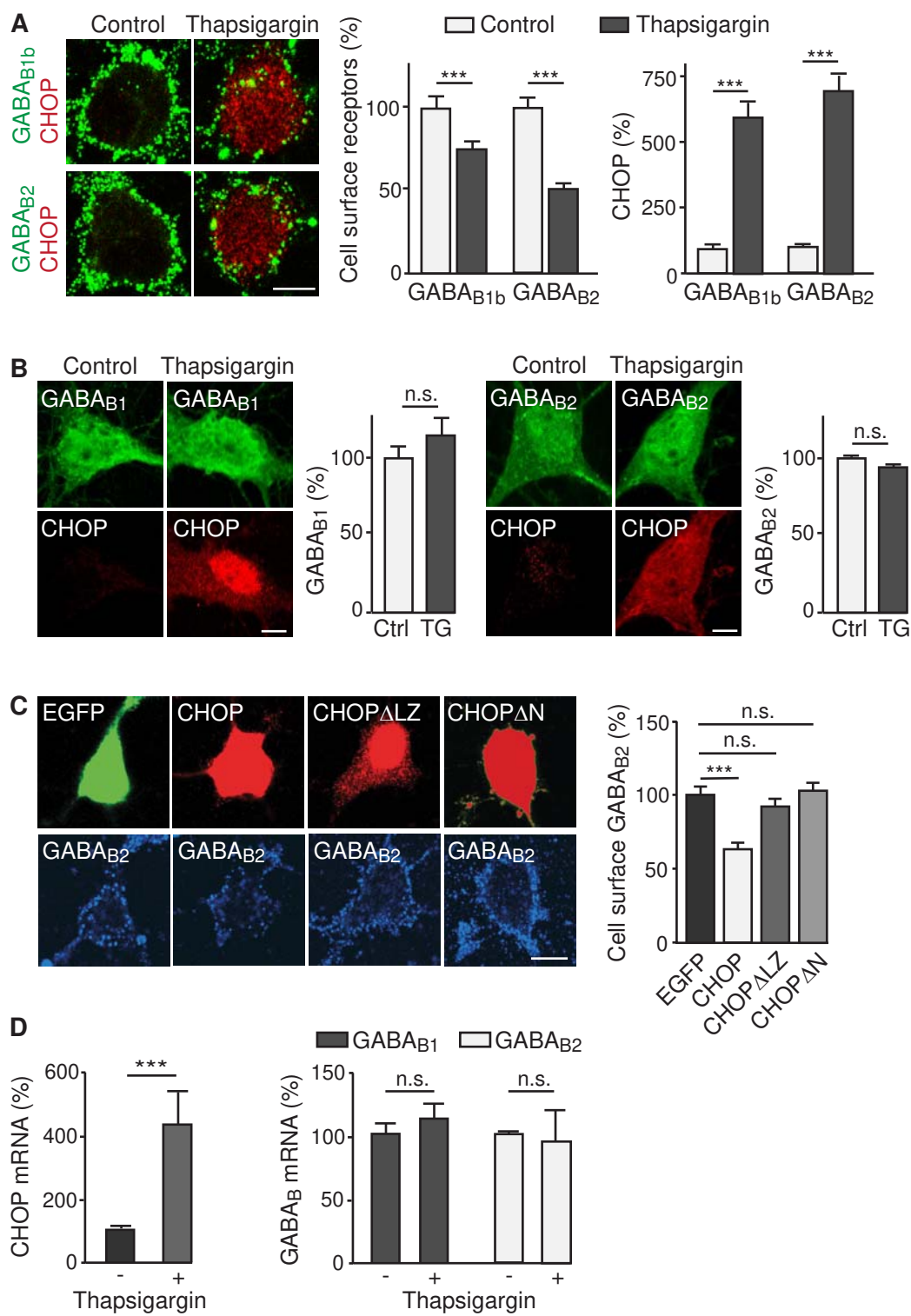


Fig. 2

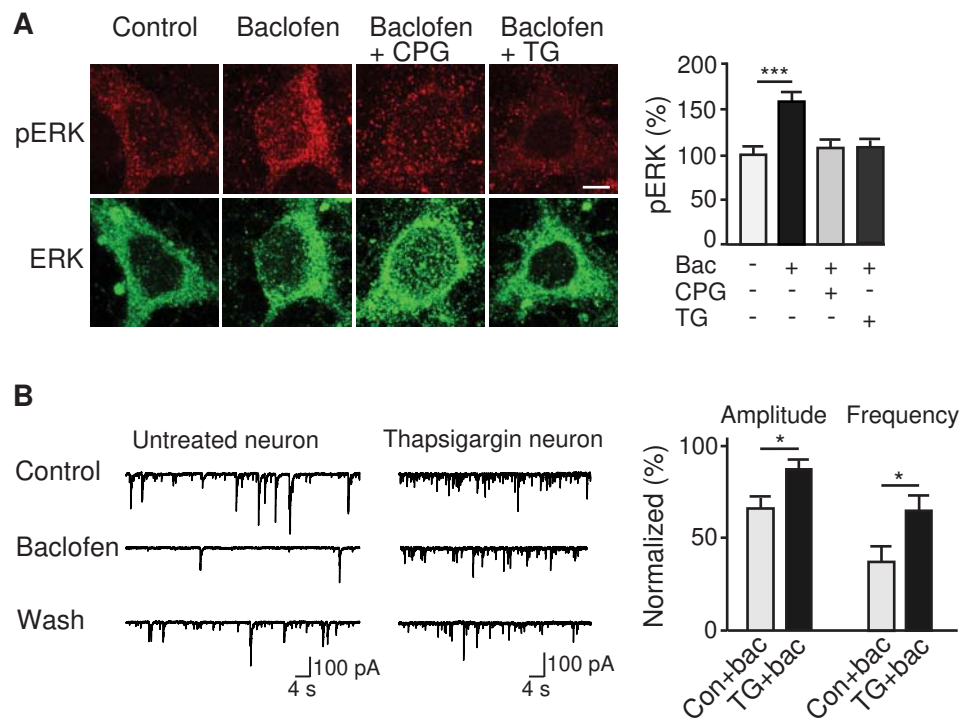


Fig. 3

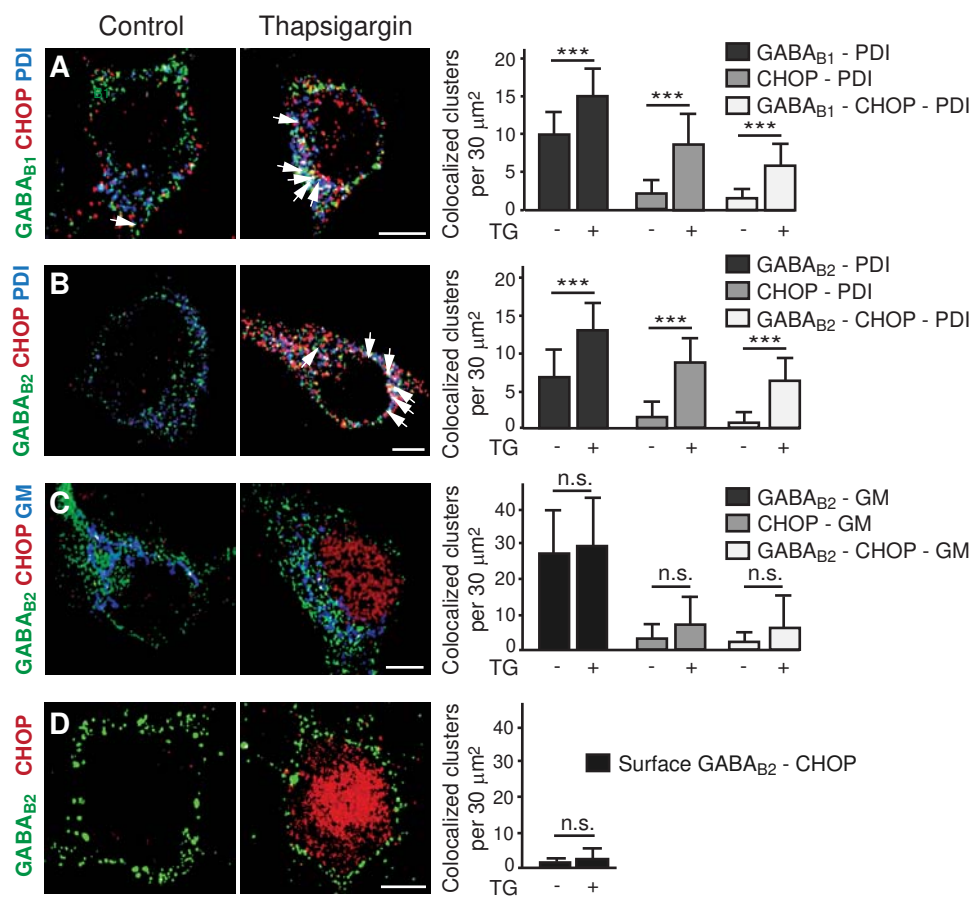


Fig. 4

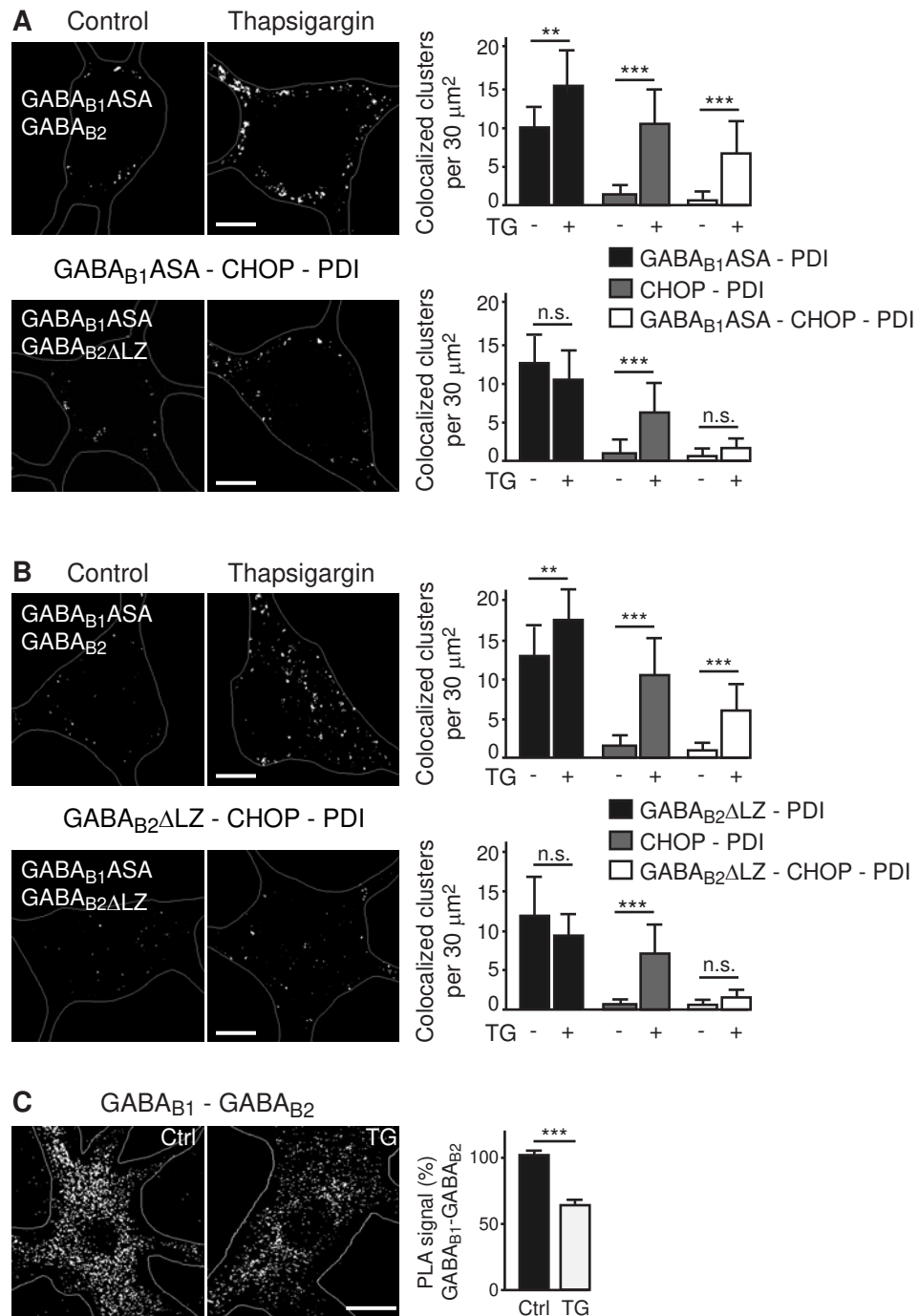


Fig. 5

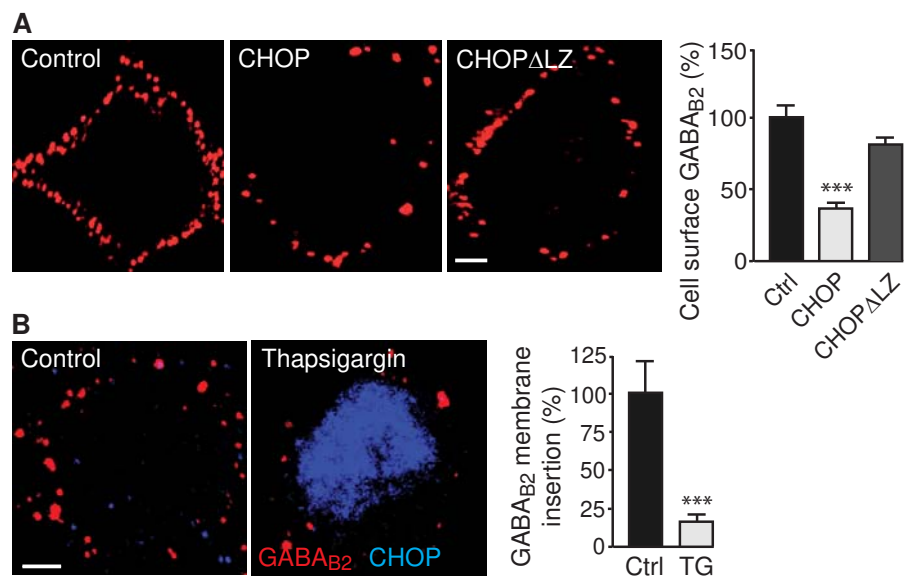


Fig. 6

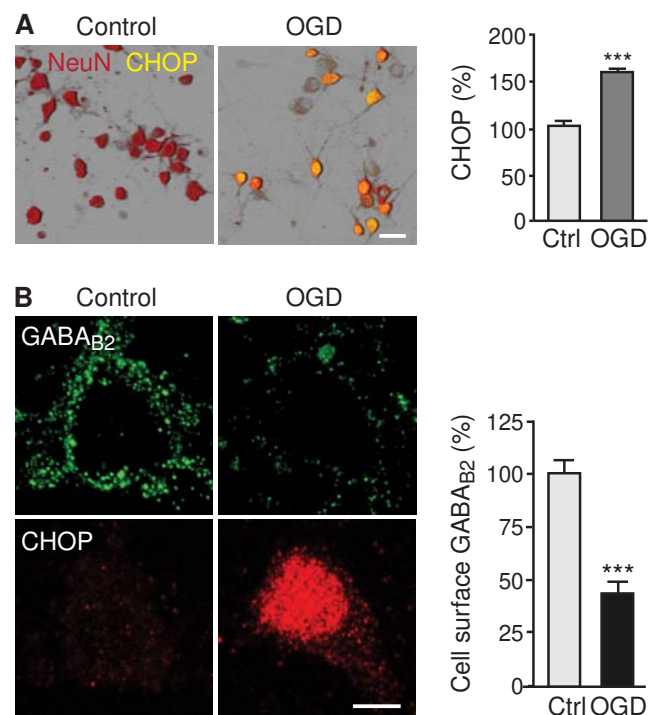


Fig. 7

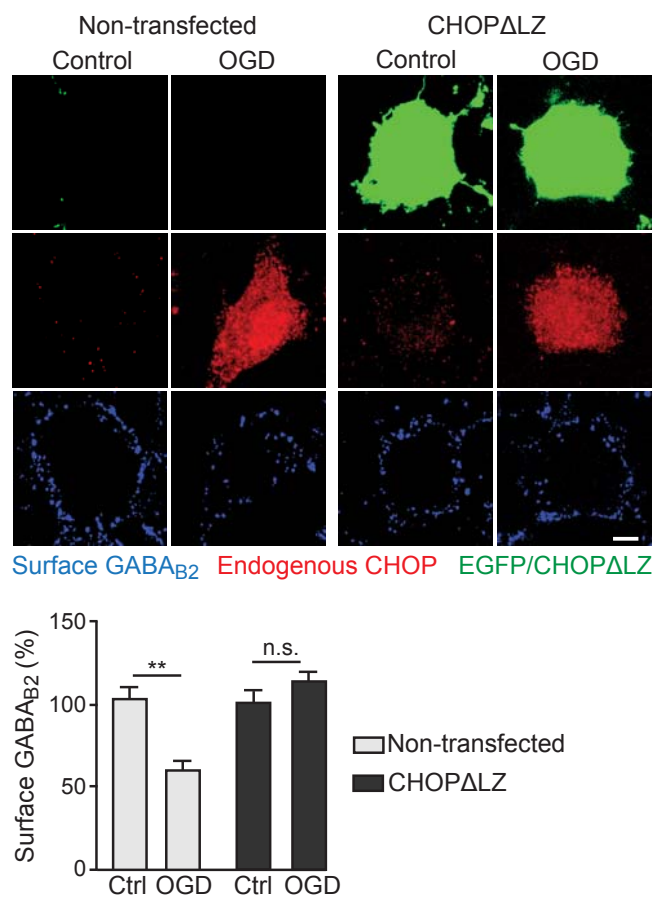


Fig. 8

Neurobiology:

**Ischemia-Like Oxygen and Glucose
Deprivation Mediates Down-regulation of
Cell Surface γ -Aminobutyric Acid_B
Receptors via the ER Stress-Induced
Transcription Factor CHOP**

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